

Relative Efficiency of Biological Transmission of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) by *Dermacentor andersoni* (Acari: Ixodidae) Compared with Mechanical Transmission by *Stomoxys calcitrans* (Diptera: Muscidae)

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ABSTRACT *Anaplasma marginale* Theiler is a tick-borne intraerythrocytic rickettsial pathogen of cattle that also can be mechanically transmitted by biting flies. Rickettsemia during the acute phase of infection may reach as high as 10^9 infected erythrocytes (IEs) per milliliter of blood. Animals that survive acute infection develop a life-long persistent infection that cycles between $10^{2.5}$ and 10^7 IE/ml of blood. We compared stable fly *Stomoxys calcitrans* (L.)-borne mechanical transmission during acute infection with Rocky Mountain wood tick, *Dermacentor andersoni* Stiles-borne biological transmission in the persistent phase of infection to demonstrate quantitatively that biological transmission by ticks is considerably more efficient than mechanical transmission by biting flies. Stable flies that partially fed on an acutely infected calf and were immediately transferred to susceptible calves to complete their bloodmeals failed to transmit *A. marginale*. Ticks that fed on the original acquisition host after it reached the persistent phase of infection (>300 -fold lower rickettsemia) successfully transmitted *A. marginale* after transfer to the same calves that failed to acquire infection after fly feeding. Failure of fly-borne mechanical transmission at a rickettsemia >300 -fold higher than that from which ticks transmit with 100% efficiency demonstrates that tick-borne biological transmission is at least 2 orders of magnitude more efficient than direct stable fly-borne mechanical transmission.

KEY WORDS mechanical transmission, biological transmission, ticks, stable flies, *Anaplasma marginale*

Anaplasma marginale Theiler is a tick-borne rickettsial pathogen of cattle that also can be transmitted mechanically by biting flies and fomites (Ewing 1981, Potgieter et al. 1981, Hawkins et al. 1982). After transmission to cattle, *A. marginale* undergoes sequential cycles of invasion, replication, and release from erythrocytes. During acute infection, cell-associated rickettsemia may reach 10^9 infected erythrocytes (IEs) per milliliter of blood. This results in clinical anaplasmosis that is characterized by anemia, weight loss, abortion, and in many cases, death. Animals that survive acute infection maintain a life-long persistent infection characterized by repeated cycles of rickettsemia ranging from $10^{2.5}$ to 10^7 IE/ml, below the threshold for microscopic detection in blood smears (French et al. 1998). Persistently infected cattle are clinically healthy and serve as reservoirs of the pathogen.

Biological transmission by ticks is believed to be necessary for transmission from persistently infected carriers because it is not dependent on the level of rickettsemia in the host. Within the tick, *A. marginale* replicates both within the gut epithelium and in the salivary gland acini, culminating in levels of 10^4 – 10^5 organisms per salivary gland during subsequent transmission feeding (Lohr et al. 2002, Futse et al. 2003). Replication within the tick results in similar high levels of *A. marginale* in the salivary gland regardless of the rickettsemia level in the blood during acquisition feeding (Eriks et al. 1993). In contrast, transmission by biting flies is presumed to be purely mechanical and thus directly dependent on the level of rickettsemia during feeding. Because of this, fly-borne mechanical transmission is thought to be possible only during the acute phase of infection. Although mechanical transmission of *A. marginale* by biting flies is commonly assumed to be a component of the epidemiology of anaplasmosis in some areas of the United States, neither the quantitative parameters of fly-borne transmission nor its efficiency relative to tick-borne transmission have been reported.

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In a review, Ewing (1981) lists at least 12 species of biting fly that had been shown to mechanically transmit *A. marginale*, including *S. calcitrans*, eight species of Tabanidae, and three species of Culicidae. However, many of the early fly transmission studies suffer from methodological problems such as inability to determine with certainty whether the recipient calves were previously infected, use of splenectomized acquisition hosts resulting in abnormally high rickettsemias, use of unnaturally large numbers of flies, and inadequate control of blood contamination. These problems cast some doubt on the validity of the results. In more recent experiments Potgieter et al. (1981) demonstrated transmission by stable flies, *Stomoxys calcitrans* (L.), and Hawkins et al. (1982) demonstrated transmission by four species of *Tabanus*. Splenectomized cattle were used as acquisition hosts in both of these studies, resulting in rickettsemias well above those that would be seen in naturally infected, spleen-intact animals. Although use of splenectomized animals demonstrates the potential for transmission, it also produces an unrealistic bias in favor of transmission.

Determinants of fly-borne transmission include feeding behavior that facilitates blood transfer from an infected to a naive animal, the proportion of fed flies that carry *A. marginale*-infected blood on their mouthparts, and the number of *A. marginale* infective units acquired and maintained in a viable state before feeding on a second host. To address these questions and to compare the efficiency of fly-borne transmission with tick-borne transmission, we used the stable fly *S. calcitrans*. Stable flies are cosmopolitan blood-feeding flies; they can reach very high population densities in areas around confined cattle operations such as feedlots and dairies. Stable flies inflict a painful bite that results in many interrupted feedings due to host-defensive behaviors. Only 27% of stable flies complete their bloodmeal on a single host (Schofield and Torr 2002). When blood feeding is interrupted, flies may immediately contact another nearby host because cattle under fly feeding pressure tend to cluster closely together. The high percentage of interrupted feedings and subsequent rapid movement between hosts provides an ideal opportunity for mechanical transmission of *A. marginale* from infected to uninfected hosts.

The minimal infectious dose (MID) of *A. marginale*-infected erythrocytes needed for mechanical transmission has not been precisely established, but it is in the relatively narrow range between the theoretical minimum of 1 IE and 100 IE that have been shown to consistently transmit infection after direct inoculation (G.H.P., unpublished observations). Estimates of the amount of blood transferred on stable fly mouthparts range from 0.029 nl (Weber et al. 1988) to 0.4 nl (Kloft 1992). At an MID of 100 IE, rickettsemias between 2.5×10^8 (or $10^{8.4}$) IE/ml and 3.5×10^9 (or $10^{9.5}$) IE/ml would be required for a single fly to transmit 1 MID. With quantitative polymerase chain reaction (PCR), transmission success or failure can be defined in quantitative terms that should improve our understanding of fly-borne transmission of *A. marginale*.

The Rocky Mountain wood tick, *Dermacentor andersoni* Stiles, is the predominant biological vector of *A. marginale* in the intermountain west. This tick transmits *A. marginale* efficiently, even at very low rickettsemias (Eriks et al. 1993). Because *A. marginale* replicates to high levels both in the gut and in the salivary glands of competent tick vectors, regardless of the rickettsemia level in the blood during acquisition feeding, the amount of *A. marginale* that can be transferred by a tick is not limited by the rickettsemia of the host at the time of tick feeding. Furthermore, much larger amounts of *A. marginale* are likely to be transferred in saliva from infected tick salivary glands than can be mechanically transferred on contaminated fly mouthparts.

In this work, we have examined the potential for mechanical transmission of *A. marginale* by stable flies by using quantitative PCR to estimate the amount of inoculum on the mouthparts of flies fed at differing levels of acute rickettsemia. By comparing mechanical transmission by stable flies during the acute phase of infection with biological transmission by *D. andersoni* during the persistent phase of infection, we have established a minimum difference in the efficiency of biological versus mechanical transmission.

Materials and Methods

***A. marginale* Strains.** Two different strains of *A. marginale* were used in these experiments. The Florida strain is nontransmissible by *Dermacentor* ticks (Wickwire et al. 1987). The St. Maries strain originated from ticks collected from a naturally infected bull in St. Maries, ID (Eriks et al. 1994) and is transmissible by *D. andersoni* (Eriks et al. 1993, Futse et al. 2003). Both *Anaplasma* strains were used for the studies to quantify the amount of *A. marginale* on the mouthparts. The St. Maries strain was used for the transmission trials.

Stable Flies. Stable flies were obtained from a colony maintained in the Department of Entomology, Kansas State University, Manhattan, KS, established from wild flies collected in Manhattan in 1990. Flies were reared as described previously (McPheron and Broce 1996) and pupae were shipped overnight to the Animal Disease Research Unit in Pullman, WA. Pupae were placed in 30-cm square cages (BioQuip, Gardena, CA) and held at 25°C and 98% RH for emergence. Adult flies were provided Gatorade ad libitum on cotton pads. The Gatorade was withdrawn 6–12 h before blood feeding.

Rocky Mountain Wood Ticks. Ticks used in these studies were obtained from a laboratory colony of *D. andersoni* established from ticks originally collected at the Reynolds Creek watershed in Owyhee County, southwestern Idaho. Colony ticks were maintained as describe by Stiller et al. (2002). These ticks have been kept in colony for >10 yr (20+ generations), and ticks from this colony have been shown to be competent vectors of the St. Maries strain of *A. marginale* (Eriks et al. 1993, Futse et al. 2003).

Cattle. All cattle used in these experiments were cared for following procedures approved by the University of Idaho Institutional Animal Care and Use Committee. Before the study all calves were confirmed to be serologically negative for *A. marginale* by using a commercially available competitive enzyme-linked immunosorbent assay (cELISA) (VMRD, Inc., Pullman, WA). Two Holstein calves were used as acquisition animals. Calf c950bl was inoculated with the Florida strain of *A. marginale* on 22 January 2003 and calf c995bl was infected with the St. Maries strain of *A. marginale* by tick passage from 14 May to 21 May 2003. The transmission studies were conducted using uninfected Holstein calves c983bl, c985bl, c989bl, and c990bl at \approx 5–6 mo of age.

Acquisition by Stable Flies Feeding during Acute Infection. Flies were fed en masse on infected calves during the acute phase of infection to determine the proportion that acquired mouthpart and gut contamination. The calf infected with the Florida strain of *A. marginale* was fed on by stable flies on 11, 21, and 25 February 2003 corresponding to 20, 30, and 34 d post-infection (PI). The calf infected with the St. Maries strain was fed on by flies on 12 and 19 June 2003 corresponding to 22 and 29 d after ticks were removed. Blood was collected from the calves on the day of fly feeding, and the percentage of parasitized erythrocytes (ppe) was determined using Giemsa-stained blood smears. For feeding, flies were placed in 0.5-liter paper cartons screened at each end with nylon tulle fabric (i.e., bridal veil). Groups of 50–70 flies were fed by holding the feeding cages against the shaved side of the *A. marginale* infected calves for 10 min. Two cages were fed simultaneously. Flies in one cage were immediately frozen on dry ice after feeding. Flies in the other cage were held off the host for 20 min after feeding before being frozen. From 4 to 21 flies per feeding were dissected; the mouthparts and guts were tested by PCR to determine the amount of *A. marginale* present. Logistic regression was used to determine the relationship between the ppe and the proportion of flies with infected mouthparts. The effect of *Anaplasma* strain and the effect of holding flies on the relationship were examined using covariates.

Fly Transmission Trial. The transmission feeding trial was conducted on 19 June 2003 by using the calf infected with the St. Maries strain as the acquisition animal. An en masse feeding trial was carried out concurrently on the same animal. Trials were conducted using flies fed in cages affixed to the backs of acquisition and transmission calves with contact cement. The cages were 13 cm in diameter by 25 cm in length screen tubes wrapped around 1-mm-thick plastic rings for support. Orthopedic stockinet was fastened at each end of the tube to allow access to the flies inside the cage. The cage was glued to a shaved area on the backs of the calves. Flies were able to feed on the animal through an 8 by 8-cm square opening on the bottom of the cage (Lysyk 1991). The length of time required to complete a bloodmeal was determined by observing flies placed into a feeding cage on an uninfected host. Feeding was timed from the point

when the fly first bit until it lifted its mouthparts and stopped feeding. The average duration of feeding was 147 s ($n = 20$; maximum 248 s, minimum 42 s, SD = 62 s), identical to the 147-s average feeding time reported by Schofield and Torr (2002). As a result, flies used in transmission feedings were allowed to feed for \approx 60 s on the calf acutely infected with the St. Maries strain of *A. marginale*, and the feeding was then interrupted by aspirating the fly into a tube. The fly was immediately transferred in the aspirator to an uninfected host and allowed to continue feeding. Flies were observed to ensure that they reinitiated feeding on the second host. This was repeated for three flies on each of two calves (c983bl and c989bl) and for 30 flies on each of two additional calves (c985bl and c990bl). Flies were frozen on dry ice immediately after their transmission feeding for later dissection.

Flies from the transmission trial were dissected and tested for *A. marginale* by PCR as described below. Guts were tested to confirm feeding on the infected host; mouthparts were tested to determine whether they retained any *Anaplasma* after the transmission feed. Flies were held frozen at -30°C before dissection and thawed on ice a few at a time for dissection. Each fly was dissected on a fresh piece of dental wax with clean forceps. The mouthparts, including the labella, labium, and a portion of the rostrum were removed dry, and then a drop of Hanks' balanced salt solution (Sigma, St. Louis, MO) was added and the gut floated out. Guts were rinsed in a drop of clean saline to remove surface contamination. The mouthparts and gut were placed individually in 100 μl of proteinase K buffer (0.01 M Tris, pH 7.8, 0.005 M EDTA, and 0.5% SDS) with 100 $\mu\text{g}/\text{ml}$ enzyme ($2\times$) and stored frozen until DNA preparations were made.

Calves were monitored for the development of *A. marginale* infection for 84 d after the fly transmission feedings by weekly examination of Giemsa-stained blood smears, by nested PCR and by cELISA (VMRD Inc.).

Tick Transmission. During feeding, male ticks were confined under cloth feeding patches as described previously (Scoles et al. 2005). Ticks were acquisition fed for 7 d (3–10 September 2003) on the St. Maries strain-infected calf c995bl, after it had entered the persistent phase of infection (112 d PI, 35 d after it first dropped below microscopic detection). The ticks were removed and held off the host for 24 h at 25°C and 98% RH before being transmission fed for 7 d on the same four calves that failed to acquire infection when stable flies were fed on them 84 d previously. Three ticks each were fed on calves c983bl and c989bl, and 30 ticks each were fed on calves c985bl and c990bl (the same calves that had earlier been transmission fed upon by three and 30 stable flies respectively). A sample of ticks was dissected before transmission feeding and a sample of the ticks from each calf was dissected after the transmission feeding. The guts and salivary glands of the transmission ticks were placed individually in 100 μl of proteinase K buffer with $2\times$ enzyme, as described above, and stored frozen until DNA preparations were made. After tick transmission

feeding calves were monitored for infection with *A. marginale* as described previously.

DNA Preparation and PCR. Tubes with dissected fly (mouthparts and gut) or tick (gut and salivary gland) tissues were thawed and incubated in the proteinase K solution at 50°C for 1 h. IsoQuick (Orca Research, Bothel, WA) lysis solution (100 μ l) was added, and the tissues were ground with a disposable plastic pestle (Bel-Art Products, Pequannock, NJ). After grinding, tubes were incubated for an additional hour at 50°C. DNA was extracted from the lysate by using the IsoQuick DNA extraction kit (Orca Research) following the modified protocol of Schwartz et al. (1997) with one additional modification. Before the DNA precipitation step, 1.0 μ l of a 20 mg/ml solution of glycogen (Roche Diagnostics, Indianapolis, IN) was added to the fly mouthpart and the tick salivary gland samples to improve the precipitation of small quantities of DNA. DNA was resuspended in 30 μ l of double-distilled H₂O and stored in the refrigerator until use.

Fly and tick tissues were tested for *A. marginale* by using a nested PCR that targets the *A. marginale* *msp5* gene. The PCR test was modified from Torini de Echaide et al. (1998) by the use of an additional primer to make the hemi-nested PCR described by the authors fully nested (Scoles et al. 2005). Based on repeated PCR amplification of the serially diluted cloned *msp5* fragment described below, we determined that the sensitivity threshold for this nested PCR is <10 copies.

Blood samples from the acquisition hosts, a sample of the tick salivary glands, and all of the fly mouthparts that were positive for *A. marginale* by nested PCR were tested by quantitative PCR by using the TaqMan protocol described by Futse et al. (2003), with the exception that the standard curve was determined using a partial *msp5* fragment (the 525-base PCR product from the external primer pair described above) that had been TA cloned into the PCR4 cloning vector (TOPO TA cloning kit for sequencing, Invitrogen, Carlsbad, CA). Samples were run in triplicate and expressed as the mean for the three replicates. The detection threshold for this quantitative PCR assay is between 10 and 10² copies. The standard curve was constructed using dilutions from 10⁶ to 10³ copies. Concentrations of samples with <10³ copies per milliliter were determined by extrapolation; samples predicted to be above 10⁶ (i.e., most blood samples) were diluted 1:10 and 1:100 so that the results would fall within the range of the standard curve.

As a control for the quality of the DNA preparation all samples that were PCR negative for *A. marginale* were tested by PCR for the presence of fly or tick DNA. Fly samples were tested using universal insect 16S rDNA primers 16Sbr: 5'-CCG GTT TGA ACT CAG ATC ATG T-3' (modified from primer LR-J-12887 by substitution of T for C at the underlined positions to match the *Aedes albopictus* (Skuse) and *Drosophila yakuba* Burla sequences) and 16Sar (LR-N-13398): 5'-CGC CTG TTT AAC AAA AAC AT-3' (Simon et al. 1994). This primer pair produces a fragment of \approx 550

bases by using an annealing temperature of 55°C. Tick samples were tested using mitochondrial 16S rDNA primers 16s+1 and 16s-1 as described previously (Norris et al. 1996).

Results

Acquisition by Stable Flies Feeding during Acute Infection. The proportion of flies that acquired detectable levels of *A. marginale* on their mouthparts after feeding on acutely infected calves increased with increasing ppe in the blood of the acquisition calf during acute rickettsemia. The logistic regression was $P(\text{infection}) = 1/(1 + \exp(-(-2.55 + 0.28 \times X)))$ where $P(\text{infection})$ is the probability of a fly having infected mouthparts and X is ppe in the blood ($\chi^2 = 16.8$, df = 1, $P < 0.0001$). At high ppe of 9.7%, as many as 55% of flies had detectable levels of *A. marginale* on their mouthparts after feeding. At intermediate ppe of 4.9–5.4%, mouthpart contamination rates ranged from 8.3 to 42.9%. There was no detectable mouthpart contamination at the low ppe (0.3–0.5%) that would be characteristic of the very early or very late phases of the acute infection (Table 1). Although the proportion of flies that had detectable *A. marginale* on their mouthparts was consistently lower in the groups held off the host for 20 min after feeding, this effect was not statistically significant ($\chi^2 = 2.1$, df = 1, $P > 0.15$). The logistic regression also did not vary between *Anaplasma* strains ($\chi^2 = 0.8$, df = 1, $P > 0.38$). Many mouthpart samples that were nested PCR positive for *A. marginale* were below the detection threshold for quantitative (real-time) PCR (Table 1).

The number of *A. marginale* copies per mouthpart for flies fed en masse, at all time points, either immediately after feeding or after holding off the host for 20 min, was low. Of the 42 flies with *A. marginale*-positive mouthparts, none were within the range of the real-time PCR standard curve (10³–10⁶ copies) (Table 1). Thus, the levels of *A. marginale* on positive mouthparts is greater than or equal to 10¹ (the detection limit for nested PCR) but <10³ (lower limit for most accurate detection by quantitative PCR). *A. marginale* was detectable on the mouthparts only of flies fed when calves had levels of 4.7×10^8 copies/ml (10^{8.67}) or greater. There was no discernible relationship between strain and the detection of *A. marginale* on the mouthparts.

Fly Transmission Trial. Neither the calves fed on by groups of three flies (c983bl and c989bl) nor the calves fed on by groups of 30 flies (c985bl and c990bl) developed *A. marginale* infection during the 84 d post-transmission observation period; *A. marginale* was undetectable on stained blood smears examined weekly or by nested PCR on days 25, 32, and 82 postfeeding. The animals were seronegative when tested by cELISA on days 67 and 84 postfeeding. At the time of the fly acquisition feeding the ppe of c995bl was 4.9% on stained blood smears; quantitative PCR indicated that there were 5.8×10^8 (10^{8.76}) genome copies per milliliter of blood. Overall, >84% of the guts of the flies dissected after the transmission feeding were nested

Table 1. Levels of *A. marginale* rickettsemia in cattle and infection of stable flies fed en masse

| Date | Rickettsemia in cattle | | Stable fly infection | | | | | | |
|---------------------------------|------------------------|------------------------|----------------------|----------------|----------------|---------------------------|---------------------|----------------|-------------------------|
| | ppe ^a | copies/ml ^b | Held ^c | n ^d | X ^e | % mouthparts ^f | % guts ^f | n ^g | Copies/fly ^h |
| Florida strain - calf C950b1 | | | | | | | | | |
| 11 Feb. | 0.3 | 1.49 × 10 ⁷ | No | 9 | 0 | 0.0 (0.0) | 100 | ND | ND |
| 21 Feb. | 9.7 | 5.36 × 10 ⁸ | No | 20 | 11 | 55.0 (11.1) | 100 | 2 | 8.95 × 10 ¹ |
| | | | Yes | 20 | 9 | 45.0 (11.1) | 100 | 1 | 3.30 × 10 ¹ |
| 25 Feb. | 5.4 | 4.72 × 10 ⁸ | No | 21 | 9 | 42.9 (10.8) | 100 | 1 | 1.68 × 10 ² |
| | | | Yes | 20 | 6 | 30.0 (10.2) | 100 | 4 | 4.70 × 10 ² |
| St. Maries strain - calf C995b1 | | | | | | | | | |
| 12 June | 0.5 | 5.79 × 10 ⁷ | No | 4 | 0 | 0.0 (0.0) | 100 | 0 | BT |
| | | | Yes | 6 | 0 | 0.0 (0.0) | 100 | 0 | BT |
| 19 June ⁱ | 4.9 | 5.80 × 10 ⁸ | No | 21 | 6 | 28.6 (9.9) | 100 | 3 | 2.22 × 10 ² |
| | | | Yes | 12 | 1 | 8.3 (8.0) | 100 | 0 | BT |
| 3–10 Sept ^j | | 1.84 × 10 ⁶ | | | | | | | |

Numbers in parentheses are standard errors of the percentages. BT, below threshold of detection; ND, not determined.

^a ppe, percentage of parasitized erythrocytes, the percentage of total erythrocytes that are infected.

^b Copies/ml is the number of genome copies determined by quantitative PCR.

^c Held indicates whether flies were held 20 m off the host after feeding.

^d n is number of flies tested.

^e X is number of flies with nested PCR-positive mouthparts.

^f The % mouthparts and % guts are the proportions that were nested PCR positive.

^g n is number of flies with quantifiable *A. marginale*.

^h Copies/fly is the number of genome copies on the fly mouthparts determined by quantitative PCR.

ⁱ Flies fed en masse at the same time as the fly transmission trial.

^j Tick acquisition feeding.

PCR positive for *A. marginale*, confirming that they had fed on the acquisition host, and importantly, the guts of 100% of the flies transmission fed in groups of three were PCR positive (Table 2). Of the 10 transmission flies with PCR negative guts, only two seemed to have no blood when dissected, indicating that they had not fed.

In the group of flies fed en masse on calf c995bl at the same time as the transmission feedings and dissected without having the opportunity to refeed, 29% (six of 21) had detectable *A. marginale* on their mouthparts (Table 1). In contrast, only 3% (two of 65) of the mouthparts of flies tested after transmission feeding, had detectable *A. marginale* (Table 2). The difference is statistically significant (*G* test of goodness-of-fit for single-classification frequency distributions with Williams' correction for sample sizes <200; *G*_{adj} = 9.3693, significant at *P* < 0.01) (Sokal and Rohlf 1987).

Tick Transmission Trial. At the time of tick acquisition feeding on c995bl infection was undetectable on

stained blood smears; quantitative PCR indicated that there were 1.84 × 10⁶ (10^{6.26}) genome copies per milliliter of blood, a 315-fold reduction in rickettsemia between the fly transmission and the tick transmission trial. A sample of ticks dissected after acquisition feeding but before transmission feeding had a 96.3% salivary gland infection rate (26/27). Real time quantitative PCR was preformed on the salivary glands of a sample of six of the transmission ticks after the transmission feeding; the average was 6.8 × 10⁶ genome copies per salivary gland pair (*n* = 6; *s* = 3.66 × 10⁶; max = 1.17 × 10⁶; min = 8.62 × 10⁴). All four of the transmission calves became infected after the tick transmission feeding. Cattle followed a normal course of infection with prepatent periods ranging from 19 to 27 d posttick attachment (Table 3); all four cattle were seropositive when tested 47 d post-tick attachment. There was no meaningful relationship between the number of transmission feeding ticks and the prepatent period, or the peak level of rickettsemia (Table 3).

Table 2. Infection of stable fly mouthparts and guts determined after stable fly transmission feeding

| Calf | No. flies | <i>A. marginale</i> infection | | | | | |
|--------|-----------|-------------------------------|---|-----------------|-----|----|-----------------|
| | | Mouthparts | | | Gut | | |
| | | n | X | % infected (SE) | n | X | % infected (SE) |
| C983bl | 3 | 3 | 0 | 0.0 (0.0) | 3 | 3 | 100.0 (0.0) |
| C989bl | 3 | 3 | 0 | 0.0 (0.0) | 3 | 3 | 100.0 (0.0) |
| C985bl | 30 | 30 | 2 | 6.7 (4.6) | 31 | 27 | 87.1 (6.0) |
| C990bl | 30 | 29 | 0 | 0.0 (0.0) | 29 | 23 | 79.3 (7.5) |

Flies were acquisition fed on c995bl during the acute phase of infection (5.8 × 10⁸ *A. marginale* genome copies per milliliter) and immediately transferred to susceptible transmission calves. *n* is number of flies examined; *X* is number of flies infected.

Discussion

The failure of stable flies to transmit *A. marginale* from an acutely infected calf, followed by successful tick-borne transmission from the same calf after it had reached the persistent phase of infection, demonstrates that tick-borne biological transmission is considerably more efficient than fly-borne mechanical transmission. As few as three *D. andersoni* were able to efficiently transmit *A. marginale* after feeding at a rickettsemia >300-fold lower than the level at which fly-borne mechanical transmission failed with three or 30 flies. This represents a minimum difference in ef-

Table 3. Infection of ticks by *A. marginale* after tick transmission feeding

| Calf | Ticks | Guts | | | Salivary glands | | | Prepatent period ^a | Peak rickettsemia ^b |
|---------------------|-----------------|------|----|-----|-----------------|----|------|-------------------------------|--------------------------------|
| | | n | X | % | n | X | % | | |
| Prefed ^c | 27 | 27 | 27 | 100 | 27 | 26 | 96.3 | | |
| C983bl | 3 | 3 | 3 | 100 | 3 | 3 | 100 | 19 | 5.40 |
| C989bl | 3 | 3 | 3 | 100 | 3 | 3 | 100 | 27 | 4.30 |
| C985bl | 30 | 16 | 16 | 100 | 16 | 16 | 100 | 19 | 7.90 |
| C990bl | 30 ^d | 16 | 16 | 100 | 16 | 16 | 100 | 25 | 8.60 |

Ticks were acquisition fed on c995bl for 7 d during the persistent phase of infection (1.84×10^6 *A. marginale* genome copies per milliliter) and then transferred to calves for 7 d transmission feeding. n is number of ticks dissected; X is number of ticks positive.

^a Days to first detection of infected erythrocytes on Giemsa-stained blood smear.

^b Peak percentage of infected erythrocytes counted on Giemsa-stained blood smear.

^c Ticks that were dissected before transmission feeding and were not transmission fed.

^d Twenty-eight ticks recovered.

iciency between mechanical and biological transmission of more than 2 orders of magnitude.

Based on quantitative PCR, we estimated that the volume of blood retained on the mouthparts of a stable fly is 0.38 nl (2.22×10^2 copies per mouthpart/ 5.8×10^8 copies per ml = 3.83×10^{-7} ml), remarkably close to the estimate of 0.4 nl (4.0×10^{-7} ml) made by Kloft (1992) but approximately 10-fold larger than the estimate of 0.0294 nl (2.94×10^{-8} ml) made by Weber et al. (1988). Kloft's estimate was based on transfer of fluid labeled with a radioisotope, whereas the estimate of Weber et al. (1988) was based on morphological examination of dried mouthparts prepared for scanning electron microscopy. The estimate of Weber et al. (1988) was indirect, relying on size calculations and assumptions about which areas might harbor intact blood cells. Our estimate, and that of Kloft, was based on empirical observations. This, and the fact that the two estimates are in relatively close agreement suggests that the larger figure, 0.4×10^{-6} ml (0.4 nl), is more likely to be correct.

Using quantitative PCR we were able to determine the number of *A. marginale* genome copies per ml of blood. Because *A. marginale* replicates within the erythrocytes after invasion the number of genome copies per erythrocyte may range from one to as many as eight (Ristic and Watrach 1963), although French et al. (1998) estimated that, on average, each IE contained two to four organisms (i.e., two to four genome copies). Using this estimate as a correction factor, the number of IE/ml can be approximated from the quantitative PCR data. The MID for *A. marginale* in cattle has been estimated to be ≤ 100 IE/ml (Palmer, unpublished observations). Assuming transfer of 0.4 nl of blood, each bite should have transferred between 58 and 116 IE at the level of infection in the calf on the day of the fly transmission feeding, at a minimum, two fly bites should have been sufficient to transfer a MID.

There are at least two possible explanations why neither three nor even 30 fly bites were sufficient to result in transmission. PCR detects *A. marginale* DNA,

not viable organisms; *A. marginale* on the mouthparts may have lost viability either from desiccation, or from exposure to some component of the saliva of the flies. Infections achieved using 100 infected erythrocytes were done using immediate dilution and transfer of infected blood using intravenous inoculation (G.H.P., unpublished observation). Thus, the relationship between PCR quantified *A. marginale* and number of viable, infectious organisms may well differ between direct inoculation versus fly-borne mechanical transmission. A second possible explanation may be that, although the mouthparts are contaminated with sufficient IE to result in infection, transfer is inefficient and at least a portion of the IE remains on the mouthparts after the transmission feeding. These two mechanisms could act separately or in concert to drastically reduce the efficiency of stable fly-borne mechanical transmission. A portion of our data can be used to argue against the inefficient transfer hypothesis. There was a significant reduction in the proportion of flies with detectable *A. marginale* on their mouthparts after transmission feeding. The significantly smaller proportion of flies with mouthpart contamination after their transmission feeding is most likely the result of the infected blood being cleansed from the mouthparts during the transmission feeding; up to 55% of nontransmission-fed flies had *A. marginale* on their mouthparts, compared with only 3% of flies after transmission feeding.

The proportion of flies with measurable amounts of *A. marginale* on their mouthparts was highest in the groups that were fed on calves with the greatest number of infected erythrocytes, represented as ppe, during acute rickettsemia. *A. marginale* was undetectable on the mouthparts of flies fed on calves with low ppe. Holding flies for 20 min after feeding consistently reduced the proportion of flies with measurable amounts of *A. marginale* on their mouthparts. Although the reduction was not statistically significant, and nothing is known about the viability of *A. marginale*-infected blood exposed to the environment, it would seem that immediate movement of flies from one host to another has a higher probability of transferring more organisms than would delayed transfer (Table 1).

Although tick-transmissible and nontick-transmissible strains of *A. marginale* may interact differently with mechanical vectors, these experiments were not designed to examine these potential differences. Differences between strains that might have an effect on mechanical transmission could include adaptations that improve pathogen survival on the mouthparts, such as desiccation resistance or resistance to degradation by components of fly saliva. They also might include adaptations for increased colony size within each erythrocyte (i.e., larger copy number per cell), which would increase the number of organisms without increasing the number of infected erythrocytes, possibly increasing the infectivity of each individual infected erythrocyte. Follow-up experiments will be needed to examine the possibility that tick-transmis-

sible and nontick-transmissible strains interact with mechanical vectors in different ways.

For this study, we choose to use a nonsplenectomized rather than a splenectomized acquisition host for two reasons: 1) the design included allowing the animal to survive to the persistent phase of infection, and 2) the flies were exposed to a more realistic level of peak rickettsemia. Because we used a spleen intact calf in these experiments, the peak rickettsemia of the acquisition host was somewhat lower ($10^{8.76}$) than could have been achieved with a splenectomized animal ($>10^9$). We also chose to use spleen intact rather than splenectomized transmission hosts to more accurately represent what would be occurring in the field. With access to sensitive tests to detect infection (nested PCR and cELISA), it is no longer necessary to use splenectomized animals to detect infection as was routinely done in the older studies.

Although the potential of fly species other than *S. calcitrans* to serve as mechanical vectors of *A. marginale* has not been investigated quantitatively, horse flies and mosquitoes have been implicated as mechanical vectors of *A. marginale* (Ewing 1981). Because blood volumes are greater and the size and morphology of tabanid mouthparts provide much more surface area for contamination, tabanid-borne mechanical transmission is likely to be more efficient than stable fly-borne mechanical transmission. Studies to quantify the efficiency of tabanid-borne mechanical transmission will be the subject of follow-up studies.

In conclusion, although this study certainly does not rule out the possibility of stable fly-borne mechanical transmission, we have clearly demonstrated that mechanical transmission by direct transfer of flies from an infected to a susceptible host is at least 2 orders of magnitude less efficient than tick-borne biological transmission. Because fly-borne mechanical transmission of *A. marginale* is generally believed to be epidemiologically important in some areas, additional studies will be necessary to clarify the conditions under which mechanical transmission occurs, and the role that it plays in the epidemiology of *A. marginale*.

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